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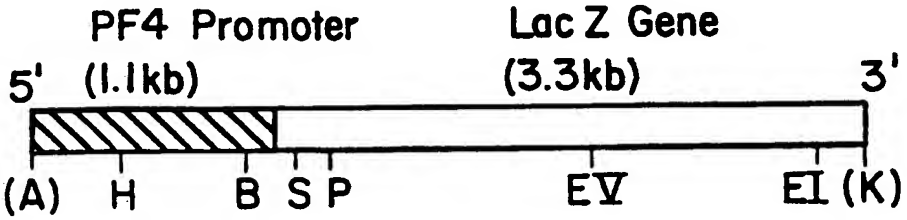

(57) Abstract

A transgenic model for selective targeting of a gene product by means of a megakaryocyte promoter, which is linked to a gene encoding the product, and a construct which includes the megakaryocyte promoter and the gene. Expression of the gene product has been shown to be limited to platelets and bone marrow megakaryocytes. In particular, the platelet factor 4 (PF4) promoter has been shown to result in targeting of expression of a gene to platelets, bone marrow megakaryocytes and, in addition, the adrenal gland.

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TRANSGENIC MODEL FOR SELECTIVE TARGETING OF GENE PRODUCTS WITH A MEGAKARYOCYTE PROMOTER

Description

Background

Pluripotent stem cells of the bone marrow give rise to the 2N megakaryoblast, which is then converted to the mature polyploid megakaryocyte. These transformations are associated with three striking cellular alterations. First, specialized membrane receptors such as GpIb and GpIIb/IIIa arise which can interact with specific plasma components and adhesive macromolecules (Hawiger, J., Steer, M., and Salzman, E. (1987) Intracellular Regulatory Processes in Platelets in R. Colman et al (eds) Hemostasis and Thrombosis. 2nd Edition. J.B. Lippincott. Philadelphia and Toronto). Second, cytoplasmic alpha granules appear, which contain hemostatic system mediators such as platelet factor 4 (PF4) and growth factors such as platelet derived growth factor (Odell, T.T. Jr. and Jackson, C.W. (1968) Blood 32, 102-110; Paulus, J.-M. (1970) Blood 35, 298-311; Ebbe, S. and Stohlman, F.Jr. (1965) Blood 26, 20-35; Oyo, R., Nakeff, A., Huang, S.S., Ginsberg, M., and Deuel, T.F. (1983) J. Cell Biol. 96, 515-520; Waterfield M.D., Scrace G.T., Whittle N., Stroobant P., Johnson A., Wasteson A., Westermark B., Heldin C.H., Huang J.S., and Deuel T.F. (1983) Nature 304, 35-39). Third, signalling pathways develop which generate cyclic nucleotides, inositol phosphates, and endoperoxides, whose summed effect

induces release of granule constitutents as well as activation of membrane receptors (Hawiger, J. et al., "Intracellular Regulatory Processes in Platelets", In: R. Colman et al. (eds.), Hemostasis and Thrombosis (2d Ed.), J.B. Lippincott, Philadelphia and Toronto (1987)). The mature polyploid megakaryocytes then fragment by a poorly understood process into large numbers of small anucleate platelets which possess all of the above cellular machinery and circulate in the blood.

Blood platelets play a central role in normal hemostasis and wound healing. The complex interactions between specific receptors, signalling pathways, and cytoplasmic granules allow platelets to bind to adhesive proteins exposed on damaged blood vessels, complex with plasma components that accelerate blood coagulation, release alpha granule constitutents which modulate blood clotting as well as stimulate wound healing, and take part in plasma protein mediated platelet-platelet aggregate formation which seals damaged areas of the vascular tree (Hawiger, J., Steer, M., and Salzman, E. (1987) Intracellular Regulatory Processes in Platelets in R. Colman et al (eds) Hemostasis and Thrombosis. 2nd Edition. J.B. Lippincott. Philadelphia and Toronto; Sixma, J.J. and Wester, J. (1977) Semin. in Hematol. 14, 265-299). Hyperactivity of the above system is believed to initiate thrombogenesis and atherogenesis, which represent the twin pathophysiologic mechanisms which produce cardiovascular disease.

The events that govern in vitro conversion of stem cells to mature megakaryocytes and platelets have been thoroughly investigated, but the in vivo regulation of

this process is poorly understood (Vinci, G., Tabilio, A., Deschamps, J.F., Vanttacke, D., Henri, A., Guichard, J., Tettroo, P., Lansdorp, P.M., Hercend, T., Vainchenker, W., and Breton-Gorius, J. (1984) Br. J. Haematol. 56, 586-605). Extensive biochemical studies have uncovered a wealth of details about how platelets are able to carry out their biologic functions under in vitro conditions, but less is known about the importance of these mechanisms under in vivo conditions.

Summary of the Invention

The present invention relates to a method of selective targeting of a gene product through the use of a megakaryocyte promoter; constructs useful in the method of selective targeting; transgenic mammals in which a megakaryocyte promoter selectively targets a gene product to bone marrow megakaryocytes, circulating platelets and the adrenal gland; and a method of treating or preventing a condition or disease in which the level of a critical component of megakaryocytes or platelets is altered by selectively augmenting or suppressing the level of the component. It further relates to a method of assessing the function of a critical component of megakaryocytes or platelets by altering its level and determining the effects of the modification.

The present invention provides a means of targeting a selected substance, such as a diagnostic, therapeutic or preventive substance, to any area of the body at which a nucleic acid sequence encoding the selected substance is delivered and expressed under the control of a megakaryocyte promoter. As described herein, platelets, bone

marrow megakaryocytes and the adrenal gland have been shown to selectively contain the product of a gene coupled to the platelet factor 4 promoter and, thus, each can be targeted as a site for expression of a nucleic acid sequence encoding a selected substance.

Therefore, the present invention is useful, for example, as a means of targeting delivery of a selected substance, such as a growth factor or tissue plasminogen activator (tpa), by means of platelets containing a nucleic acid sequence encoding the selected substance, to an area to which platelets migrate in vivo (e.g., an affected area such as a wound or site of a myocardial infarct). In addition, the present invention provides a method of targeting to the adrenal gland a selected substance, such as a glucocorticoid or other drug beneficial to the adrenal gland. Further, it is possible to use the present method in treating conditions in which megakaryocyte function is altered, such as in megakaryocyte leukemia.

Brief Description of the Drawings

Figure 1 is a 1181 bp nucleotide sequence of the rat PF4 gene. The transcription start site is indicated in a box; the GATAAA sequence is underlined; and the sequence linked to the HGH gene in PPF4GH is indicated by an arrow.

Figure 2A is a schematic representation of the PF4lacZ construct, which contains 1.1 kb of the 5′ upstream region of the rat PF4 promoter linked to the 3.34 kb of the prokaryotic β-galactosidase (lacZ) gene without its regulatory region. Abbreviations used: A,

ApaI cohesive ends; EI, EcoRI; EV, EcoRV; H, HindIII; K, KpnI cohesive ends; P, PstI; and S,SphI.

Figure 2B is a photograph of a Southern blot of mouse DNAs prepared from F2 offspring of founder 10. Lanes 1,2: homozygotes; lanes 3,5: nontransgenic mice; lanes 4, 6, 7 and 8: heterozygotes. λHindIII markers are indicated by arrows.

Figure 3 shows expression of the PF4lacZ construct in hematopoietic cells. In situ staining for prokaryotic β-galactosidase was carried out on a peripheral blood smear of a transgenic mouse (A); purified platelet fractions from a transgenic mouse (B) or a normal litter mate (not shown); bone marrow from a transgenic mouse (C) or a normal litter mate (D); splenic tissue section from a transgenic mouse (E); and thymic tissue section from a transgenic mouse (not shown). Magnification: (A)x400; (B) x1000; (D)x100; and (E) x40.

Figure 4 shows expression of the PF4lacZ construct in adrenal gland from a transgenic mouse. In situ staining for prokaryotic β-galactosidase was carried out on tissue sections of the adrenal gland from a transgenic mouse (A) and a normal litter mate (not shown). Magnification: x40.

Detailed Description of the Invention

It has been discovered, as described herein, that a tissue specific promoter can be used to target delivery to and expression of a selected substance or gene product to a tissue or tissues. In particular, it has been shown that a megakaryocyte promoter coupled to a nucleic acid

sequence encoding a selected substance is useful for selective expression of the encoded substance in platelets, bone marrow megakaryocytes and the adrenal gland. As described herein, the 5′ upstream region of the platelet factor four (PF4) promoter has been coupled to a gene encoding a selected product and expressed in a transgenic mammal. As further described herein, in the transgenic mammal, platelets were the only circulating blood cells and megakaryocytes were the only hematopoietic precursor cells in which the selected product occurred. The selected product was also evident to a lesser extent in the adrenal gland, in which its distribution corresponded to the location of mineralocorticoid secreting cells. The megakaryocyte promoter described is the PF4 promoter, which has a high power of expression in cells of this type. Thus, in addition to specific targeting, it offers the advantage of a high level of expression of a protein in platelets. However, another megakaryocyte promoter, such as the IIb gene promoter, can be used to target genes of interest to megakaryocytes and circulating platelets. The 5′ upstream region of the PF4 promoter used contains most, if not all, of the tissue specific region. Figure 1 shows a 1181 bp nucleotide sequence of the rat PF4 gene. Transient expression experiments which are described in greater detail in Example 1 revealed a complex interplay between a core promoter domain from -97 to the transcriptional start site, and an enhancer silencer domain from -448 to -112.

The core promoter contains a GATA site at -31 to -28 whose mutation to TATA or AATA decreases tissue specificity and moderately affects expression in megakaryocytes as well as a postive-acting subdomain from -97 to -83 whose removal decreases overall transcription without affecting tissue specificity. The enhancer/silencer domain possesses three positive-acting subdomains from -380 to -362, -270 to -257 and -237 to -120 as well as a negative-acting subdomain at -184 to -151 which is able to reduce overall transcription but has no effect on tissue specificity. The subdomain -380 to -362 is most critical in restricting gene expression driven either by the PF4 promoter or by a heterologous promoter to the megakaryocytic lineage. The subdomains -270 to -257 and -137 to -120 function together with subdomain -380 to -362 to somewhat increase tissue specificity. Simultaneous mutation of the GATA site and deletion of either the whole enhancer/silencer domain or subdomains -380 to -362 or -137 to -120 reduce transcription in megakaryocytes to 10-30 fold. Based upon the above results, the megakaryocyte-specific enhancer/silencer domain and the GATA site are likely to be responsible for high level expression of the PF4 gene in a lineage-specific manner.

The PF4 promoter region can be linked to a nucleic acid sequence(s) or gene(s) of interest (i.e., encoding a selected product(s) and used to produce transgenic mammals or introduced into an individual (prenatally or postnatally). For example, a PF4 promoter region-nucleic acid sequence construct can be produced and introduced, as described herein, at an embryonic stage, such as into the pronuclei of fertilized eggs, which are reimplanted

into a female and maintained under appropriate conditions for development. The resulting offspring contain the construct and the encoded product is targeted specifically to platelets, bone marrow megakaryocytes and the adrenal gland. Alternatively, the PF4 promoter region can be linked to a nucleic acid sequence(s) or gene(s) to form a PF4 promoter region-nucleic acid sequence construct which can be introduced into bone marrow of an individual (e.g., prior to bone marrow transplantation) for selective targeting of the encoded product to platelets, megakaryocytes and the adrenal gland.

The following is a description of: 1) a construct, referred to as PF4lacZ, which includes a megakaryocyte promoter region and a gene encoding β-galactosidase and its use in producing two lines of transgenic mice which express the construct; 2) characterization of the transgenic mice, particularly as to location of expression of the construct; and 3) application of the present finding to selectively target gene products.

The PF4lacZ construct and its use in generating transgenic mice are described in detail in Example 1. Briefly, as shown in Figure 1, the PF4lacZ construct includes 1.1kb of the 5′ upstream region of the rat PF4 promoter linked to the 3.34 kb of the prokaryotic β-galactosidase (lacZ) gene without its regulatory region. A full length rat PF4 cDNA has been isolated and sequenced by Doi and co-workers. Mol. and Cell. Biology, 7:898-904(1987). The teachings of this reference are incorported herein by reference. The 1.1kb genomic PF4 fragment includes 1104 bases of the 5′ upstream sequence and the cap site to +20. This segment of the PF4 gene

was selected because transient expression assays utilizing primary bone marrow cells suggested that the tissue specific regulatory elements are located within this domain. Ravid, K. et al., FASEB J. Abstract 1392 (1990). The PF4 fragment was inserted into a PUC19-based plasmid (pSDKlacZpA) which contains the 3.34 kb prokaryotic β-galactosidase gene without its upstream regulatory region, flanked by a unique HindIII site at the 5′ end and a unique BamHI site at the 3′ end. Plasmid PF4lacZ was deposited (September 28, 1990), under the terms of the Budapest Treaty, at the American Type Culture Collection (Rockville, MD) under accession number 40903.

Production of the transgenic mice is described in Example 2, as is assessment of tissue distribution of the product (β-galactosidase). Two lines (line 10 and line 12) of transgenic mice which express the construct were generated, as explained in Example 2. Transgenic mice from both lines were assessed for expression of the PF4lacZ construct or transgene in peripheral blood cells, bone marrow progenitor cells, splenic cells, thymic cells, and cells from other organs (adrenal gland, brain, heart, intestine, kidney, liver, lung and skeletal muscle). Normal litter mates served as controls.

Assessment of blood, bone marrow, spleen and thymus showed that platelets were the only circulating blood cells and megakaryocytes were the only hematopoietic precursor cells in which β-galactosidase was detected. Results also showed the lack of transgene expression in brain, heart, kidney, intestine, liver, lung and skeletal

muscle. Unexpectedly, the adrenal gland exhibited approximately 2% of the levels of β-galactosidase of megakaryocytes. The enzyme was localized to the area of mineralocorticoid secreting cells.

As a result of the work described herein, it is possible to selectively target gene products (production of a selected substance) in circulating platelets, bone marrow megakaryocytes and the adrenal gland through use of the megakaryocyte promoter PF4. That is, as described herein, a selected nucleic acid sequence (a gene or gene fragment) is linked to a megakaryocyte promoter in such a manner that expression of the nucleic acid sequence is under the control of the megakaryocyte promoter. The resulting megakaryocyte promoter-nucleic acid sequence construct is introduced into a mammal under conditions appropriate for expression of the product encoded by the nucleic acid sequence. The construct can be used to produce transgenic animals or can be introduced into an individual (e.g., into somatic cells). The construct can be used to overexpress (cause increased expression of) a gene or nucleic acid sequence, as well as to underexpress (cause reduced expression of) a gene or nucleic acid sequence. In the former, a gene encoding, for example, a growth factor can be overexpressed. In the latter, for example, an active form of a specific gene product can be underexpressed in order to assess its effect on a wound healing model or on megakaryocyte development. This can be done by overexpressing a mutated form of a protein subunit, resulting in a significant decrease in the level of the active protein (i.e., an inactive dimer). Alternatively, an antisense gene can be overexpressed,

resulting in inactivation of a platelet or megakaryocyte gene.

The megakaryocyte promoter in the construct can be, for example, the platelet factor 4 (PF4) promoter, the IIb gene promoter region or other megakaryocyte promoter. Based on what is disclosed herein with regard to the regulatory elements of the PF4 promoter, other megakaryocyte promoters can be isolated and used to selectively target gene products as disclosed herein. The term megakaryocyte promoter as used herein means all or a portion of a megakaryocyte promoter; a promoter portion is one of appropriate size and sequence to have the desired function (ability to cause tissue specific expression of a nucleic acid sequence). The megakaryocyte promoter component of the construct can be obtained (isolated) from a naturally-occurring source or can be produced using any of a variety of techniques, such as genetic engineering or cloning methods, PCR amplification or synthetic techniques. Although the promoter described herein is of murine origin, it can be from another source (or have the sequence of a megakaryocyte promoter of other than murine origin). For example, it can be of human origin and preferably will be in those applications carried out in humans. In addition, the sequence of the megakaryocyte promoter component of the construct need not be precisely the same as that of a megakaryocyte promoter, but must only be sufficiently similar that it have the desired function for which a megakaryocyte promoter is used (i.e., tissue selective expression of a selected gene or nucleic acid sequence). The gene(s) or selected nucleic acid

sequence(s) in the construct encode one or more products whose expression in platelets and megakaryocytes is desired. In the case of the PF4 promoter, the gene(s) or selected nucleic acid sequence(s) can also be targeted to the adrenal gland. The gene or nucleic acid sequence can be obtained (isolated) from a naturally-occurring source or can be produced using any of a variety of techniques, such as genetic engineering or cloning methods, PCR amplification or synthetic techniques. For example, a gene encoding tissue plasminogen activator (tpa) can be incorporated into a construct of the present invention and used to treat or prevent occlusions, such as occur in myocardial infarcts. Alternatively, a gene encoding a growth factor can be incorporated into a megakaryocyte promoter-nucleic acid sequence construct and introduced into an individual to enhance wound healing or vascular proliferation. A key advantage to expression of a selected product such as tpa or a growth factor in platelets is that platelets migrate to the affected areas (e.g., wound, occlusion) and, as a result, will concentrate delivery of the encoded product at the site or sites at which it is needed, rather than introducing the product in a more systemic manner. A megakaryocyte promoter-growth factor construct can also be used to produce an immortalized cell line of megakaryocytes, depending on the growth factor and the sensitivity of the megakaryocyte to the factor being overexpressed. A particular advantage to the present invention is that it provides a means of targeting a selected product to platelets, which contain coagulation

factors which accelerate blood clotting, adhesive proteins which facilitate the interactions of platelets and other blood cells and growth factors which stimulate the process of wound healing. Another advantage to use of a construct of the present invention is that it provides a means by which continuous production of the encoded substance is possible. That is, because the gene encoding the selected product is present in a cell, it will continue to be expressed for the life of the cell. A further advantage to use of a construct of the present invention is that expression of the particular product can be controlled (i.e., can be turned on or off) by manipulating the regulatory elements. For example, expression can be turned off by mutating the GATA site and/or deleting positive regulatory domains as as P_1 (-137 to -120), P_2 (-270 to -257) and P_3 (-380 to -362).

The construct of the present invention can be used to produce transgenic animals, in which it is expressed on an ongoing basis and transmitted to offspring. Alternatively, a construct can be introduced into an individual in need of therapy or preventive treatment. For example, a construct can be introduced into bone marrow megakaryocytes, using known techniques, such as a retroviral vector. As a result, platelets produced will contain the construct, express the encoded product and serve as a production and delivery system for the product.

Transgenic animals in which the megakaryocyte promoter-nucleic acid sequence construct results in production of a reporter gene can be used in developmental biology experiments. For example, an oncogene can

be introduced into an animal model expressing a reporter gene, such as β-galactosidase, driven by the PF4 promoter to analyze the effect of the insertional locus of these oncogenes on the loss of differentiation and tissue specific expression.

The transgenic mouse produced as described herein, in which the PF4 promoter drives a marker gene (e.g., the prokaryotic β-galactosidase gene) can be used to assay the effect of agents on megakaryocyte differentiation and platelet formation, by means of detecting β-galactosidase activity. For example, an in vitro assay can be carried out to assess the effects of different agents, such as thrombopoietin (McDonald, T. P. , "Thrombopoietin: Its Biology, Purification and Characterization", Exptl. Hematology, 16 (1988), on megakaryocytes in cultured bone marrow. In this assay, β-galactosidase serves as a selectable marker for megakaryocytes. Alternatively, an in vivo model can be used for assessing platelet production and megakaryocyte ploidy by assaying for β-galactosidase in bone marrow of transgenic mice subjected to manipulations designed to alter or influence platelet production or megakaryocyte ploidy (e.g., treatment with thrombopoietin).

Transgenic animals can also be produced using a construct in which an oncogene is under the control of the megakaryocyte promoter. For example, a megakaryocyte promoter-oncogene construct can be introduced, as described herein, to produce transgenic mice from which immortalized adrenal cortex cells can be obtained. In one embodiment, the PF4 promoter can be linked to the temperature sensitive mutated form of the large T antigen

and introduced into mice, producing transgenic mice. A cell line of immortalized megakaryocytes can be produced by obtaining bone marrow cells into which the oncogene-containing construct has been introduced and growing them in vitro at a permissive temperature. Such immortalized megakaryocyte cell lines can be used, for example, in in vitro study of megakaryocyte differentiation.

This invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

Example 1. Transient Assay Technique

Materials: Propidium iodide, Bothrops jararaca snake venom, adenosine, sodium citrate and theophylline (Sigma, St. Louis, MO); DEAE-Dextran (Pharmacia, Piscataway, NJ); (alpha^{32}P) ATP, and (alpha^{35}S) ATP (New England Nuclear Research Products, Boston, MA); Disposable 1 ml cuvettes (Sarstedt, Boston, MA); Nylon mesh screens (Spectramesh, Spectrum Medical, Inc., Los Angeles, CA); Granulocyte-macrophage colony stimulating factor (GM-CSF) (Genzyme Corporation, Boston, MA); Erythropoietin (Amgen Biologicals, Thousand Oaks, CA); Human growth hormone (HGH) radioimmunoassay kit (Nichols Institute, San Juan Capistrano, CA); Restriction enzymes (New England Biolabs, Beverly, MA); DH5 alpha competent bacteria (Bethesda Research Laboratories, Bethesda, MD); Erase-a-Base System (Promega, Madison, WI); Culture media and sera except for rat plasma (Gibco Laboratories, Grand Island, NY); Sprague-Dawley-derived (CD) rats (Charles

River Breeding Laboratories, Wilmington, MA). Oligonucleotides employed for sequencing and internal deletion or mutation experiments were synthesized with beta-cyanoethylphosphoamidites on a DNA synthesizer (model 8600, Biosearch, Inc., San Rafael, CA).

Plasmids: pOGH and pSVTKGH were a generous gift from R.F. Selden, Boston, MA. pPF4GH was constructed by digesting a plasmid containing the rat PF4 gene (Doi, T. et al., Mol. Cell. Biol. 7:898-904 (1987)) with EcoRI and BamII, ligating the resultant fragment spanning the 5′ upstream region from -1104 to +20 with the synthetic linkers.

AATTGCACATATGTGC and CAAGTCGACTTGGGCT
CGTGTATACACGTTAA TCGGGTTCAGCTGAAC

respectively, digesting the product with NdeI and SalI, and then ligating the above segment with the NdeI/SalI cut pOGH which contains the 4.5 Kb fragment with the HGH gene. The control plasmid for the HIRT assay (see below) was generated by linearizing pOGH with HindIII, treating the linearized plasmid with alkaline phosphatase, and then ligating a 6.5 Kb HindIII fragment of the human thrombomodulin (TM) gene into this site.

The 5′ deletions of the pPF4GH were carried out with the Erase-a-Base system. The plasmid was digested with NdeI and 5′ protruding ends were filled with Klenow fragment as well as alpha-thiodeoxynucleotide triphosphates. The resultant fragment was then cut with StuI at a site ~170 bp downstream of the 5′ end of the sequence, and digested for varying times with exonuclease III. The

nucleotide sequences of deleted mutants were determined by the dideoxy sequencing method. The 5' deletant from -273 was specifically constructed by removing the NdeI to AccI (-255) fragment of pPF4GHd (see below) and then inserting synthetic DNA to replace -272 through -255 as well as restore the NdeI site.

The internal deletions, replacements, and mutations of pPF4GH were mainly carried out with PCR generated fragments utilizing as template M13mp19 viral DNA containing the HindIII (PF4) to SacI (GH) fragment (M13-PF4GH). PCR was conducted in 50 uM KCl and 1.5 to 3 mM $MgCl_2$ in 10 mM Tris-HCl, pH 8.3, with 200 μM dNTPs and 1 μM primers. The reactions were performed for 20-25 cycles of 1 min at 94°, 1 min at 42°-55°, and 2 min at 72°C. The integrity of product ends was insured by incubating with 5 units of Klenow for 15-20 min at 24°. The structures of the fragments were confirmed by dideoxy sequencing. In some cases, a HindIII (-735) to XbaI (-20) fragment was subcloned into pBluescript [pBSrPF4(H-X)] since the BglII site at -122 and the BamHI site at -222 are unique. In most instances, the modified fragments were eventually exported to pPF4GHd. pPF4GHd was obtained by removing adapter and polylinker sequences between the rat promoter and the GH gene of pPF4GH such that nucleotide +16 of the rat gene is linked to +2 of the GH gene which provided a unique XbaI site at -20.

The pPF4GH sequence from -444 to -112 was deleted by digesting a bBluescript clone containing a SmaI fragment of rPF4 (-844 to -59) with SfaNI (-449), blunting with Klenow, and cutting with HindIII (-736). The rPF4 HindIII to SnaNI fragment was isolated and then ligated

with pPF4GH from which the HindIII (-735) to ScaI (-112) fragment had been removed by partial ScaI digestion. The pPF4GH GATA sequence from -31 to -28 was mutated to AATA or TATA by carrying out PCR with a sense primer spanning -265 to -249 and a degenerate antisense primer spanning -18 to -40 with A/T replacing G at -31 in conjunction with the template M13-PFGH. The product was self ligated, digested with BgIII and XbaI, and ligated with pBS rPF4 (H--X) from which the BgIII to XbaI rPF4 fragment had been excised. Recombinants were identified by sequencing, and HindIII to XbaI fragments carrying AATA and TATA sequences were exported back tp pPFGHd. The TATA mutation of the GATA sequence was also prepared within the pPFGH deletant at -97 by partially digesting the plasmid with XbaI (2 sites 49 bp apart) as well as BspEll and then removing the small fragments generated. The resultant cut plasmid was then ligated to the BspEll to XbaI fragment of the PF4 promoter carrying the TATA/AATA mutations. The BspEll to XbaI fragments containing the TATA/AATA mutations were also used to replace native fragments in pPF4GHducta. The pPF4GH sequence from -97 to -83 was deleted by performing inverse PCR on a self ligated 940 bp AccI fragment derived from pPF4GHd with a sense primer spanning -82 to -67 and antisense primer spanning -98 -115. The product of the expected size was self ligated, giested with BgIII and XbaI and ligated into pBSrPF (H-X) from which the corresponding fragment had been excised. The HindIII to XbaI fragment of a recombinant confirmed by sequencing was exported back to pPF4GHd. The pPF4GH sequence from -137 to -116 was deleted and an AfIII site created by

carrying out PCR employing an antisense primer spanning -96 to -115 as well as -138 to -157 and a sense primer spanning -463 to -445 in conjunction with the M13-PF4GH template. The product was digested with BamHI (-222) and ScaI (-112), and the resultant fragment between these two sites was isolated. The PBSrPF4 (H-X) was digested completely with BamHI and partially with ScaI (2 sites), the product singly cut at the rPF4 ScaI site was purified, and then ligated with the above PCR fragment. The HindIII to XbaI fragment was then exported to PF4GHd. The pPF4GH sequence from -212 to -149 was deleted by partially digesting pPF4GHd with BamHI (-222) as well as BglII (-122), and then isolating the resultant product of the largest size. The excised segment was replaced with a synthetic oligonucleotide of 45 bp which resulted in the removal of nucleotides -212 to -149, their replacement with AGCTGGTAC, and the introduction of unique NheI and KpnI sites. The above construct was cut with KpnI, treated with alkaline phosphatase and ligated with polynucleotide kinase treated duplex $C(AT)_{13}GGTAC$. The pPF4GH sequence from -270 to -257 was deleted by carrying out PCR employing an antisense primer spanning -236 to -256 as well as -271 to -290 and a M13 reverse sequencing sense primer in conjunction with the M13-PF4 template. The resultant product was digested with HindIII and AccI and ligated to pPFHGH pre-cut in the same sites (pPF4GHdc). To facilitate insertion of DNA at the PF4 AccI site, a second AccI in the second intron of HGH was removed (pPF4GHdca). The pPF4GH sequence from -380 to -362 was deleted by carrying out PCR employing an antisense primer comprised of the last five nucleotides of a

SnaBl recognition site and -381 to -395 and a sense primer spanning -361 to -345 in conjunction with the self ligated BgIII (-756) to BamHI (-222) fragment template derived from pPFGHd. The PCR products of the expected sizes were isolated, self ligated and digested with HindIII and Accl. The resultant products were ligated with vector prepared by removing the HindIII to Accl fragment from pPFGHdca. Recombinants were identified by the appearance of a SnaBl site (pPF4GHdua). The sequences from -137 to -116 and -270 to -257 were deleted by digesting the pBSrPF4 (H-X) containing the -137 to -116 deletion with HindIII and BamHI. The HindIII to BamHI fragment was replaced with the corresponding fragment from pPF4GHdc and the HindIII to Xbal fragment from the resulting recombinants was exported back to pPF4GHd (pPF4GHdct). The pPF4GH sequences from -270 to -257 and -380 to -362 were deleted as outlined for -380 to -362 except that the BgIII to BamHI template was derived from pPF4GHdc (pPF4GHduca). The pPF4GH sequences from -137 to -116, -270 to -256, and -380 to -362 were deleted by removal of the Accl to Xbal fragment from pPF4GHduca and its replacement with the corresponding fragment from pPF4GHdt (pPF4GHducta).

The PF4 regulatory sequences above -112 were inserted upstream of the heterologous TK promoter in order to test these segments for tissue-specific enhancer function. The pPF4GH was cut with Ndel (-1104) and Scal (-112), or SfaNl (-448) and Scal (-112) and pPF4GH -273 was cut with Ndel (-273) and Scal (-112). The various resultant fragment were blunted with Klenow, individually inserted into the unique Ndel site of pTKGH (230 bp

upstream of the TK promoter) which had been blunted as well as treated with alkaline phosphatase, and then selected for correct orientation. In similar fashion, PF4 regulatory sequences above -112 but with internal deletions were also linked to the heterologous TK promoter. To this end, pPF4GHdua, pPF4GHdct, and pPF4GHducta were cut with SfaNl (-448) as well as Scal (-112), and the resultant fragments were individually inserted into pTKGH as outlined above. The PF4 enhancer domain was also inserted 1.2 kB upstream of the TK promoter. This was accomplished by ligating a 1004 bp Rsal fragment of M13 gene IV to either end of a blunted SFaNl to Scal PF4 fragment and then separately cloning the two resultant fragments into the Ndel site of pTKGH.

Cultured Cells: Rat bone marrow cells were isolated and cultured as described previously (Kuter, D.J. et al., Blood 74:1952-1962 (1989)). The bone marrow cells were grown in 5% CO_2 at 37°C in Iscove's Modification of Dulbecco's Medium (IMDM) supplemented with 200 units/ml of penicillin, 200 ug/ml of streptomycin, 0.592 mg/ml of L-glutamine, 15% rat plasma-derived serum or 20% horse serum, 1 unit/ml of erythropoietin and 10 units/ml of GM-CSF. The culture conditions were chosen to promote maximal growth of myeloid cells, erythroid cells, and megakaryocytes (Kuter, D.J. et al., Blood 74:1952-1962 (1989)). The human erythroleukemia cell line, HEL cell line, was grown as described previously (Papayannopoulou, Th. et al., Blood 72:1029-1038 (1988)). Bovine skin fibroblasts were cultured in the presence of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics.

Transfection and Transient Expression Assay Conditions: Electroporation was used for the transfection of bone marrow cells and HEL cell lines (passages 8-12) essentially as described by Narayanan et al., Biochem. Biophys. Res. Commun. 141:1018-1024 (1986). Cells were washed with the electroporation buffer (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 5 mM $MgCl_2$), centrifuged at 30 xg for 5 min, and resuspended at a density of either $25x10^6$ rat bone marrow or 10^7 HEL cells per 0.35 ml of the electroporation buffer. Plasmid DNA in a circular form was added to the cell suspension and the mixture was kept on ice for 15 min in a 1 ml disposable cuvette. Electric charge was generated at a setting of 1.745 Kv and 0.9 mA and transmitted through a platinum electrode to the ice-cold mixture of cells and DNA. Each sample was incubated on ice for 12 min, brought to room temperature for 15-20 min, washed once with 1 ml IMDM, and then cultured in a 35 mm dish.

The levels of HGH secreted by bone marrow cells or HEL cells after 72 hr in culture increase linearly as a function of plasmid concentration used for transfection, with 60 ug producing maximal transient expression. The concentrations of HGH secreted by cells transfected with 57 ug of circular pPF4GH also increase linearly as a function of time until about 96 hr in culture. Based upon the above data, a standard transient expression assay was established in which 57 ug of circular pPF4GH was transfected by electroporation into cells and the amount of HGH produced was measured in the media after 88 hr in culture. The assay conditions allow measurement of plasmid expression to be carried out within a linear range of response.

Fibroblasts were transfected by the DEAE-dextran method (McCutchan, J.H. and J.S. Pagano, J. Natl. Cancer Inst. 41:351-356 (1968)). To this end, 35 mm culture dishes were inoculated with 1-2x10^5 cells. After 24 hr, the cells were rinsed with serum-free DMEM and the DEAE-dextran mixture was added [400 ug/ml of DEAE dextran (molecular weight 500,000), 8 ug/ml of chloroquine, 25 ug/ml of circular plasmid DNA and 8 ug/ml of sonicated herring sperm DNA in 1 ml of serum-free DMEM]. After incubation at 37°C in 5% co_2 for 1 hr, the DEAE-dextran mixture was removed and 1 ml of 10% dimethyl sulfoxide (DMSO) in HEPES-buffered saline was added (Parker, B.A. and G.R. Stark, J. Virol. 31:360-369 (1979)). The cells were incubated at room temperature for 3 min, washed with DMEM three times, and cultured in 3 ml of DMEM containing 10% fetal bovine serum, 100 U/ml of penicillin and 100 ug/ml of streptomycin at 37°C in 5% CO_2 for 72 hrs.

Cell Lysis and Radioimmunoassay: The non-adhering rat bone marrow cells were removed from wells, centrifuged at 380 xg for 5 min, washed with 2 ml CATCH (Kuter, D.J. et al., Blood 74:1952-1962 (1989)), subjected either to botrocetin treatment or resuspended in 0.3 ml of H_2O, and lysed by freezing and thawing twice. The adhering cells were scraped off the culture dishes in the presence of 1 ml of ice-cold CATCH, harvested as outlined above, washed with 2 ml ice-cold CATCH, and lysed as described above. The levels of HGH expressed by the transfected cells were measured in the media and cell lysate by radioimmunoassay (Selden, R.F. et al., Mol. Cell. Biol. 6:3173-3179 (1986)). The standard curve used for HGH was linear from 0.02-0.5 ng/ml and from 0.5-50 ng/ml with a break point at 0.5 ng/ml.

Hirt Assay: The average amount of plasmid DNA transfected into the various cell types was determined by Hirt assay (Hirt, B., J. Mol. Biol. 26:365-369 (1967)) of pooled lysed adhering and non-adhering cells. The cells were lysed by incubation for 2 hr at 4°C with 0.8 ml/plate of 0.6% SDS, 0.01 M EDTA, pH 7.5, 3 mg/ml of calf DNA employed as a carrier, and 410 pg/ml of TM control plasmid used to standardize the variable recovery of transfected plasmid DNA. NaCl was then added to a final concentration of 1.0 M, and the resultant mixtures were maintained overnight at 4°C. The lysates were centrifuged at 7000 xg for 20 min at 4°C, 0.7 ml of the supernatants were treated with DNAse-free RNAse (50 ug/ml), and DNA was extracted with phenol-chloroform followed by precipitation with ethanol as well as a 70% ethanol wash. The DNA was then digested with EcoRI and quantitated by Southern blot analysis using the 1.85 kB BamHI/SphI fragment of the HGH gene as a probe (Selden, R.F. et al., Mol. Cell. Bio. 6:3173-3179 (1986)) with a Betascope 603 blot analyzer (Betagen). The results obtained from each plate were normalized to 100% recovery of the TM control plasmid.

Selective Removal of Transfected Megakaryocytes from Cell Cultures by Addition of Botrocetin: Transfected rat bone marrow cells from two plates were suspended in 1 ml of PEC (1 ml of 1.66% EDTA, 4.5 ml of CATCH, 4.5 ml of citrated rat plasma), containing 10^8 rat platelets. Aliquots of the PEC cell suspension were removed for HGH measurements or Hirt assay of whole cell populations. Botrocetin was isolated from crude snake venum as described by Read, M.S. et al., Proc. Natl. Acad. Sci.

USA 75:4514-4518 (1978)). The partially purified botrocetin (A_{280}=0.67) was added to PEC cell suspensions (50 ul/ml PEC), the mixtures were stirred for 10 min at room temperature, and aggregated megakaryocyte/platelets were selectively removed by filtration through 17 um nylon mesh. The filtrated (megakaryocyte-depleted) cells were collected by centrifugation at 380 xg for 5 min and then resuspended in H_2O for HGH determinations or in SDS buffer for Hirt assays. The number of filtrated cells was greater than 98% of the total number of cells counted prior to filtration. The supernatants of the pelleted megakaryocyte-depleted cells were also analyzed for HGH and the amounts observed were added to those found in the cell lysates. The transient expression of the various plasmids with rat bone marrow megakaryocytes was deduced by comparing results of HGH and Hirt assays for whole cell populations with those for the megakaryocyte-depleted cell populations. We attempted to recover megakaryocytes retained by the 17 um nylon mesh in order to carry out assays directly on this cell type. However, the recovery of HGH following sonication was poor while detergents interfered with the HGH assay.

Northern Blot Analysis: Total RNAs from transfected or non-transfected cells were prepared by the CsCl cushion method (Chirgwin, J.M. et al., Biochemistry 18:5294-5299 (1979)). After formaldehyde agarose gel (1% w/v) electrophoresis, RNA was transferred with 10xSSC onto GENATRAN nylon membrane (Plasco Inc., MA). The membrane was then washed with 2xSSC for 10 min and prehybridized at 65°C for 1 hr in hybridization buffer [0.125 M Na_2HPO_4(pH 7.2), 0.25 M NaCl, 7% SDS, 1 mM EDTA,

10% PEG 6000, and 100 ug/ml sonicated heat denatured herring sperm DNA]. Hybridization with random primed labeled probe was performed at 65°C overnight with gentle agitation. The membrane was washed twice with 2xSSC at room temperature for 15 min and with high salt buffer [0.125 M Na_2HPO_4(pH 7.2), 2% SDS and 1 mM EDTA] at 65°C for 30 min and finally once with low salt buffer [0.025 M Na_2HPO_4(pH 7.2), 1% SDS and 1 mM EDTA] at 65°C for 30 min.

S1 Nuclease Protection: S1 nuclease protection experiments were conducted to establish the 5′ ends of GH mRNAs generated during transient expression assays with PF4GH and its various 5′ deletants. The single stranded probe utilized for the above studies was prepared by subcloning a 1350 bp HindIII-SacI fragment of pPF4GH into M13mp19, and then isolating viral DNA. A 20 nucleotide primer was synthesized complementary to nucleotides 24 to 53 of human GH mRNA. The primer was annealed to the single stranded M13 DNA, extended by addition of DNTPs and T7 DNA polymerase, and digested with BglII (site at -117 in the PF4 gene). The resultant product was electrophoresed on a 4% polyacrylamide-8 M urea gel, the 206 bp single stranded probe was located, excised and recovered by elution. The studies to map the 5′ end of HGHmRNA were carried out as described in (Emorine, L.J. et al., Proc. Natl. Acad. Sci. USA 84:6995-6999 (1987)).

RESULTS

PF4 Promoter Expression in Megakaryocytic and Non-Megakaryocytic Cell Lines

Two segments of the rat PF4 gene containing the 5' upstream region from -1104 or -50, the CAP site, and the three exons/two introns to +1275 were cloned into pUC19. These plasmids were transiently cotransfected with pSV2NEO by electroporation into a human erythroleukemia cell (HEL) line which exhibits megakaryocytic characteristics (Papayannopoulou, Th. et al., Blood 72:1029-1038 (1988)), as well as fibroblasts which possess no such features. The HEL cells transcribe the endogenous PF4 gene as shown by Northern analyses probed with human PF4 gene sequences, while rat cDNA does not recognize the human mRNA under our hybridization conditions. The Northern analyses also show that HEL cells express the transfected rat PF4 gene extended to -1104 but not the one truncated to -50, whereas fibroblasts do not express either construct. Normal expression of the cotransfected neomycin resistance gene is observed in both cell lines. These results indicate that the 5' upstream region of the PF4 gene is mainly responsible for its tissue-specific regulation.

To identify regulatory domains of the 5' upstream region of the PF4 promoter, a chimeric gene (pPF4GH) was constructed which possesses 1104 bp of the 5' upstream region of this gene, the CAP site to +20, and the human growth hormone (HGH) gene (Tsai, S.F. et al., Nature 339:446-451 (1989)) inserted into pUC12. The HEL cells

were transfected by electroporation with pPF4GH or plasmids containing varying lengths of the 5′ upstream region of the PF4 gene, and transient expression assays for secreted HGH were carried out as outlined above. The results delineate several major potential regulatory domains which significantly modify transcriptional activity of neighboring regions (Table 1).

TABLE 1

Expression of HGH in HEL Cells and Fibroblasts Transfected with pPF4GH and Different 5′ Deletions[a]

Plasmid	HGH Secreted (ng/plate/HIRT unit)[b] HEL	Fibroblasts
SVTKGH	5.4	25.5
PF4GH	40.0	0
-451	19.1	0
-290	18.0	0
-256	2.5	0
-184	1.6	0
-151	7.1	0
-124	2.4	0
-97	15.5	2.1
-50	0	0

[a]Low passage HEL cells (p=8-10) and fibroblasts were transfected and cultured as outlined in Materials and Methods. The plasmid number indicates the 5′ end of each plasmid deletion. The levels of HGH secreted represent an average of 2 experiments. The sensitivity of the HGH assay is in the range of ≥0.05 ng/plate.

[b]The amount of plasmid detected by HIRT assay in the cells transfected with the pPF4GH was arbitrarily defined as 1 unit and the levels of the different plasmid deletions detected by HIRT assay were expressed as a function of the level obtained with pPF4GH. The amounts of secreted HGH generated by the different constructs were then divided by the relative levels of intracellular plasmid

The elimination of upstream sequences beyond -50 reduces secreted HGH from 40 ng/plate to undetectable levels, indicating that critical regulatory domains exist in the 5′ region of the gene as previously surmised from the transfection of the entire PF4 gene. Similar experiments undertaken with fibroblasts show a considerable expression of the non-tissue-specific plasmid pSVTKGH, but an undetectable expression of pPF4GH or its 5′ deletions except for the construct truncated to -97. These results suggest that potential regulatory domains above -97 are necessary for silencing the expression of the PF4 gene in fibroblasts.

S1 protection experiments were carried out with total RNA isolated from HEL cells transfected with the varying lengths of the PF4 promoter starting at -1104, -151, and -97 as well as a mock transfected control. RNA obtained from these various cells was found to protect a single stranded labeled DNA fragment of 89 bases which corresponds to the transcriptional start site of the PF4 gene. The levels of protected fragment are in excellent accord with measurements of secreted HGH carried out at the same time with the same cells.

Lineage-Specific Expression of the PF4 Promoter in Rat Bone Marrow Megakaryocytes

To further examine the tissue-specificity of the rat PF4 promoter, we transfected primary cultures of rat bone marrow cells with pPF4GH as outlined above. The short term culture system is composed of nonadhering progenitor and mature cells of the erythroid, lymphoid, myeloid and

megakaryocytic lineage as well as adhering phagocytes, epithelial cells, and adipocytic cells (Ebbe, S. and F. Stohlman, Jr., Blood 26:20-35 (1965); Martin, D.I.K. et al., Nature 334:444-447 (1990)). The megakaryocytes comprise about 1% of nonadhering cells with a polyploid distribution similar to that of normal in vivo bone marrow. The transfected non-adhering cells were placed on glass slides, treated with ^{125}I labeled anti-HGH antibody, subjected to autoradiography, and then stained for acetylcholinesterase, a specific marker of rodent megakaryocytes (Kuter, D.J. et al., Blood 74:1952-1962 (1989)). Detailed examination of several slides containing 10^5 cells revealed that 1-2% of the cells stained positive for acetylcholinesterase with about 15% possessing autoradiographic grains whereas cells which were negative for acetylcholinesterase were completely free of autoradiographic grains. These data show that pPF4GH is only expressed in megakaryocytes and not in other bone marrow cells, which indicates an exquisite restriction to a specific lineage.

A rapid technique was then developed to monitor transcription of transfected constructs in megakaryocytes. Rat bone marrow cells were transfected with pPF4GH, media as well as nonadhering and adhering cells were separately harvested, the cell populations were lysed, and the levels of HGH were determined. The amounts of HGH found within the nonadhering cells represented over 98% of HGH detected in total cells (Table 2).

TABLE 2

Lineage-Specific Expression of pPF4GH in Bone Marrow Cells[a]

	HGH EXPRESSION (ng/plate/HIRT/unito[b]				
	Total Cells			Megakaryocyte Depleted Cells	
Plasmid	Media	Adhering	Non-Adhering		(%)[c]
SVTKGH	0.46	0.130	0.142	0.216	(79.4)
PF4GH	5.00	0.040	3.020	0.230	(7.5)
None[d]		0.036	0.048		

[a]Rat bone marrow cells were transfected, cultured and depleted of megakaryocytes by botrocetin as outlined in Materials and Methods. Cells from two plates were pooled for determining the expression of each plasmid. The results provided are of a representative experiment.

[b]Units of HGH expression are as described in Table 1.

[c](HGH in megakaryocyte depleted cells/HGH in total cells) x100.

[d]Cells cultured in the presence of medium supplemented with HGH (0.5 ng/ml). This concentration of hormone was selected because it lies within the range observed with pPF4GH.

The trace levels of HGH associated with adhering cells may be due to endocytosis of hormone generated by the nonadhering cells, as suggested by the observation that non-transfected adhering cells internalized small amounts of HGH added to the incubation media (Table 2). The HGH present in the nonadhering cells was shown by differential agglutination of megakaryocytes to be located within this cell type. Botrocetin is a snake venom protein (Howard, M.A. et al., Br. J. Haematol. 57:25-28 (1984)) which selectively aggregates megakaryocytes and allows them to be separated from other nonadhering bone marrow cells. This approach permits 90% of the rat megakaryocytes to be agglutinated free of other non-adhering cells as determined by flow cytometry. Therefore, megakaryocyte-specific gene expression can be determined by comparing the amounts of HGH in nonadhering bone marrow cells to that in the same cell population following addition of botrocetin and separation of agglutinated cells by filtration through 17 μm nylon mesh. The data in Table 2 show that the megakaryocyte-depleted fraction exhibits about 80% of the HGH present within the nonadhering bone marrow cells when transfected with pSVTKGH whereas the same cell population possesses only 7.5% of the hormone present within the nonadhering bone marrow cells when transfected with pPF4GH. These results confirm the immunohistochemical study which showed a megakaryocyte-specific expression of pPF4GH.

Tissue-Specific Regulatory Domains of the PF4 Promoter Deliniated by 5′ Deletions

The regions of the PF4 gene which are responsible for regulating lineage-specific expression were initially defined by carrying out 5′ deletions of the promoter in pPF4GH. Transient expression of pPF4GH in bone marrow cells is substantial whereas removal of the upstream sequences (deletions to -50 and -12) reduces secreted HGH to undetectable levels. The transfection of rat bone marrow cells with plasmids containing varying lengths of the 5′ upstream region delineates potential regulatory domains whose removal alters transcriptional activity by 5-30 fold. These regions include positive-acting domains at -372 to -355, -272 to -257, -151 to -125, -97 to -83, and negative-acting domains at -332 to -291, -184 to -152 (thymidine cluster) and -124 to -98. The above changes in expression are not caused by the positioning of vector sequences close to PF4 domains since a similar overall pattern of HGH secretion, but with a 10 fold reduced amplitude, is noted with 5′ deleted linearized constructs containing only PF4 and HGH sequences. In this regard, S1 analyses of total RNA from bone marrow cells transfected with pPF4GH (equivalent of bone marrow from 5 rats) revealed a faint band at the correct transcriptional start site. The quantities of material required made S_1 analyses on the 5′ deletants impractical.

The botrocetin technique was used to ascertain whether the various 5′ deletions of the PF4 promoter are expressed solely in megakaryocytes. The HGH concentrations in the megakaryocyte-depleted fraction of the

same bone marrow cells are rather constant averaging about 10% of that observed with the whole cell population until the deletion reaches position -97, when a small increment of about 6% is observed. These data indicate that removal of sequences upstream of -97 allows low level HGH expression in non-megakaryocytes. These results also reveal that changes in transcriptional activity of the 5' upstream region deleted to -151 and probably -97 are due to altered expressed in megakaryocytes rather than aberrant expression in other bone marrow cells.

Comparison of the 5' upstream region of the recently published human PF4 gene (Eisman, R. et al., Blood 76:336-344 (1990)) and the rat PF4 gene shows extended sequence identities within the PF4 promoter which closely correspond to four critical subdomains identified by 5' deletions. The conserved positive regulatory subdomains are termed P_1 (-137 to -120), P_2 (-270 to -257), and P_3 (-380 to -362) and the conserved negative subdomain is designated N_1 (-180 to -155).

Upstream Domains of the PF4 Promoter Modulate Transcription from Heterologous Promoters

The results presented in Table 1 demonstrate that the PF4 gene sequence from the transcriptional start to -97 (arbitrarily termed core promoter) does not possess all the elements required for lineage-specific expression. Therefore, the intrinsic ability of PF4 upstream domains to enhance and restrict expression of a heterologous promoter to the megakaryocytic lineage was investigated by inserting these segments in both orientations

230 bp upstream of the TK promoter. The results obtained for each construct are expressed as intracellular HGH in bone marrow cells or HGH secreted by fibroblasts relative to pTKGH. The region from -448 to -112 enhances expression of the TK promoter in megakaryocytes by 4 fold. This domain also decreases expression of HGH in bone marrow cells depleted of megakaryocytes and in fibroblasts by about 3-4 fold. Therefore, this element alters overall levels of HGH in megakaryocytes relative to other bone marrow cells by about 16 fold. The region from -448 to -112 functions in either orientation or when positioned 1234 bp upstream of the heterologous promoter. Similar sets of experiments have been conducted with the CMV promoter linked to the coding region of the β-galactosidase gene with virtually identical results.

Internal deletions of the P_1, P_2 and P_3 subdomains abolish most of the tissue-specific enhancer/silencer activity of the -448 to -112 region. Removal of the P_3 subdomain and adjacent sequences (construct -272 to -112) also reduces the activity of this region. Thus, P_3 appears to be critical for the function of the enhancer/silencer domain, but subdomains P_2 and P_1 may also be important in this regard.

Interactions Between Major Domains of the PF4 Promoter

The interactions between the core promoter from -97 to the transcriptional start site, the enhancer/silencer domain from -448 to -112, and the region from -1104 to -449 were evaluated by mutations as well as internal deletions/replacements with pPF4GH. The effects of these

alterations on megakaryocyte expression were ascertained by comparing the level of HGH generated by a given plasmid in the megakaryocyte population to that produced by pPF4GH. The effects of these changes on lineage-specific expression were determined by comparing the percentage of HGH generated by a given plasmid in the non-megakaryocytic population to that in cells transfected with pSVTKGH. The latter plasmid was utilized for comparison because it is nonselectively expressed in megakaryocytic and non-megakaryocytic populations. Thus, a completely restricted expression in megakaryocytes would be indicated by zero whereas a total loss of lineage fidelity would be signified by one. The effects of these modifications on fibroblast expression were evaluated by the amount of HGH produced by a given plasmid.

The core promoter was examined by deletions/mutations in order to identify the element(s) responsible for the relatively restricted expression of plasmid -97 in bone marrow cells. Deletion of the positive-acting domain from -97 to -83 in pPF4GH reduces transcriptional activity in megakaryocytes by 3 fold but has no effect on lineage specificity. GATA sequences have been previously described as binding sites for transacting factors involved in gene regulation of hematopoeitic cells (Martin, D.I.K. et al., Nature 334:444-447 (1990)). Mutation of the GATA sequence at -31 to -28 in pPF4GH to AATA or to TATA does not significantly affect the transcription of pPF4GH in megakaryocytes but causes a partial loss of lineage specificity and low level expression in

fibroblasts. Mutation of the GATA sequence in pPF4GH deleted to -97 leads to a complete loss of lineage specificity and a significant decrease of transcription in megakaryocytes. It is also of interest to note that the same plasmid exhibits an enhanced level of expression when transfected into fibroblasts.

The enhancer/silencer domain was analyzed to confirm elements involved in tissue-specific expression. The deletion of the entire enhancer/silencer domain from -448 to -112 leads to a three fold reduced expression in megakaryocytes as well as a significant decrease in lineage-specificity and to a moderate expression in fibroblasts. This should be contrasted with the rather minor effect on lineage-specificity observed upon 5' deletion of this domain. Presumably the region from -1104 to -449 contains a non-tissue-specific regulatory sequence which might play a dominant role in gene transcription after removal of the enhancer domain. The examination of enhancer/silencer subdomains placed upstream of a heterologous promoter suggested that P3 plays an important role in lineage-specific expression. Therefore, we tested whether the effects of grossly removing the entire enhancer/silencer domain from pPF4GH are duplicated by eliminating P3. Deletion of P3 reduces expression in megakaryocytes by about two fold, but also induces transcription in other bone marrow cells, albeit, to a lesser extent than observed with gross removal of the enhancer/silencer domain. The deletion of P1, P2 and P3 as compared to removal of P3 is only moderately more effective in altering the function of the enhancer/silencer domain. As predicted, deletions of P1, P2 or P1

and P2 have only a minimal impact on lineage-specific expression. It is also of interest to note that removal of the negative-acting subdomain N1 or its replacement with $(AT)_{13}$ enhances expression by about two fold in megakaryocytes without permitting transcription in other bone marrow cells. In this context, we observe that a nuclear transacting factor termed datin which binds to poly dT:poly dA but not to poly dAT:poly dAT has been isolated from yeast (Winter, E. and A. Warshavsky, The Embo. J. 8:1867-1877 (1990)). It is possible that a protein homologous to detin may function as a suppressor of PF4 gene expression.

Whether the core promoter and the enhancer/silencer domain function independently of each other was determined by evaluating the effects of mutations in the core promoter coupled with internal deletions in the enhancer/silencer domain. The results demonstrate that mutation of the GATA sequence combined with a deletion of P3 or P1, P2, and P3 or the entire enhancer/silencer domain, compared with the same alterations in either of these domains, decreases transcriptional activity in megakaryocytes by 10-30 fold and further reduces lineage specificity. It is also of interest to note that mutation of the GATA site combined with deletion of P1 and P2, compared with the same alterations in either of these domains, reduces transcriptional activity in megakaryocytes by about 15 fold.

Example 2. Construction of Plasmid PF4lacZ

The plasmid pPF4lacZ was constructed by inserting a 1.1 kb rat PF4 NdelBan II genomic fragment which includes

1104 bases of 5′ upstream sequence as well as the cap site to + 20 into a PUC19 based plasmid designated as pSDKlacZpA (a generous gift from Dr. Janet Rossant, Mount Sinai Research Institute, Toronto, Canada). This latter plasmid contains the 3.34 kb prokaryotic β-galactosidase gene without its upstream regulatory region flanked by a unique Hind III site at the 5′ end and a unique Bam HI site at the 3′ end. The construction of PF4lacZ was accomplished by ligating the rat genomic fragment to Hind III linearized pSDKlacZpA with synthetic linkers TCGGTCGA (Ban II to HindIII) and AGCTGGGCCCCA (Ndel to HindIII)

CCCGGGGTAT

respectively, which creates a unique Apa I site at the 5′ end of the megakaryocyte promoter. The PF4 promoter/β-galactosidase gene of 4.4 kb can be removed from pPF4lacZ as a unit free of vector sequences by cutting with Apa I and Kpn I.

Example 3. Production of Transgenic Mice and Assessment of β-Galactosidase Activity

The 4.4 kb Apa I-Kpn I fragment of pPF4lacZ was isolated by agarose gel electrophoresis, purified by C-TAB (International Biotechnologies, Inc.), diluted to 2 ug/ml in 10 mM Tris buffer, pH 7.5 containing 0.25 mM EDTA, and injected into the pronuclei of fertilized eggs from superovulated female mice (FVB/ NTacfBR, Taconic) as previously described. Hogan, B. et al., Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986). Injected embryos were reimplanted into pseudopregnant outbred females (CD-1, Charles

River), and offspring were screened for transgene integration by Southern blot hybridization of tail DNA with a ^{32}P-labelled 3.34 kb prokaryotic B-galactosidase DNA probe. Two founder mice (founder 10 and founder 12) were identified out of 12 mice produced. Both founders were mated to CD-1 females to generate F1 transgenic heterozygous and homozygous mice. Founder 10 transmitted the transgene to its progeny in a Mendelian fashion. Founder 12 was presumed to be mosaic as only 1 out of 22 offspring was transgenic but subsequent inheritance was as predicted for a normal autosomal locus. Southern analyses of founders as well as F1 progeny and F2 offspring utilizing EcoRI showed a single band of 4.4 kb. Heterozygous and homozygous F2 mice were easily identified by gene dosage. Homozygosity was confirmed by mating homozygotes to nontransgenic mice and demonstrating that all offspring were transgenic. Founder 10 exhibited 2-4 copies of PF4lacZ integrated into a single chromosomal site in a head to tail fashion as judged by comparison with diluted linearized control DNAs. F1 progeny of founder 12 which inherited the transgene possessed 1 copy of PF4lacZ integrated into a single chromosomal site.

The tissue distribution of β-galactosidase was ascertained by a combination of in situ staining of isolated blood cells as well as tissues, flow cytometric analyses of bone marrow cells, and enzymatic assay of blood cells as well as tissue homogenates.

Transgenic mice as well as their normal litter mates were anesthetized with ether prior to sacrifice. The organs obtained included adrenal gland, brain, bone

marrow, heart, intestine, kidney, liver, lung, skeletal muscle, spleen, and thymus. Tissue samples were immediately fixed for 1 hr at 4°C with 2% (w/v) paraformaldehyde in 0.1 M PIPES buffer, pH 6.9, supplemented with 2mM $MgCl_2$ and 1.25 mM EGTA, incubated for 3 hr at 4°C with PBS supplemented with 2mM $MgCl_2$ and 30% sucrose, embedded in OCT (Miles, Inc.) and frozen on dry ice. Serial sections were cut with a Cryostat at a thickness of 6 micron and placed on glass slides. Alternate serial cuts were stained with hematoxylin and eosin (James, J., Light Microscopic Techniques in Biology and Medicine, The Hague, Martinuz Nijhoff (1976)) or fixed for 5 min with 0.5% glutaraldehyde in PBS at 4°C, washed with PBS containing 2 mM $MgCl_2$, incubated at 4°C for 10 min in the presence of PBS containing 2mM $MgCl_2$, 0.02% NP-40, and 0.01% sodium deoxycholate, and stained for β-galactosidase activity at 37°C for varying periods of time with X-Gal solution [35 mM $K_3Fe(CN)_6$; 35mM $K_4Fe(CN)_6.3H_2O$; 2mM $MgCl_2$; 0.02% NP-40; 0.01% sodium deoxycholate; and 1 mg/ml of X-Gal (Bethesda Research Lab)]. Prolonged staining for the enzyme (greater than 8 hr) generates positive reactions in nontransgenic tissue unless a eukaryotic β-galactosidase inhibitor such as chloroquine is employed. Therefore, all studies were carried out for 3-4 hr in the absence of chloroquine as well as for 10 hr in the presence of the inhibitor at a concentration of 0.3 mM.

Blood samples were obtained from transgenic mice and their normal litter mates by cardiac puncture. Peripheral blood cells were isolated by centrifugation

for 8 min at 800xg, platelets were harvested by centrifugation for 15 min at 3160xg, and femoral bone marrow cells were collected as previously described. Burstein, S.A. et al., Blood 54:169-179 (1979). Blood and bone marrow cells were streaked on glass slides and stained with Wright's stain. Suspended blood and bone marrow cells were also fixed for 15 min with 0.5% glutaraldehyde in PBS containing 2 mM $MgCl_2$, and stained at 37°C for varying periods of time with modified X-Gal solution [7mM $K_3Fe(CN)_6$; 7 mM $K_4FE(CN)_6.3H_2O$; 2 mM $MgCl_2$; o.o2% NP-40; and 1 mg/ml of X-Gal] in the presence and absence of chloroquine as outlined above.

Bone marrow cells that exhibit -galactosidase activity were isolated by fluorescence activated cell sorter (FACS), and the cell populations obtained were identified with respect to cell type and DNA content. To this end, $1x10^7$ bone marrow cells were cultured for 2 hr at 37°C in 5% CO_2 in 3 ml of RPMI 1640 media (Gibco) and 10% fetal bovine serum (Gibco) with 18 uM Hoechst dye added for quantitation of DNA content per cell. The cells were washed with PBS, and then prepared for measurement of β-galactosidase with the fluorescent substrate fluorescein-di-galactoside (FDG) as previously described. Nolan, G. P., et al., Proc. Natl. Acad. Sci. USA 85:2603-2607 (1988). The flow cytometry and cell sorting were carried out on a FACStar Plus (Becton Dickinson, Mountain View, CA). Illumination was provided by two 5 watt Innova 90-5 lasers (Coherent Inc. Palo Alto, CA), one tuned to the 350-360 nm line and the other tuned to the 488 nm line. The excitation light was

focused through a standard Becton Dickinson 2 inch lens, horizontally expanded. The blue fluorescence from the Hoechst dye was collected through a 450 DF20 optical filter and the fluorescein fluorescence through a 530 DF20 optical filter. The instrument was aligned prior to use, and a C.V. of better than 2.0% was routinely obtained. The β-galactosidase positive cell population was identified by employing as negative controls cells from the same transgenic animal which had not been loaded with the FDG substrate, as well as cells from normal litter mates which had been loaded with the FDG substrate. The cell population with a DNA content of 2N was identified by assuming that the majority of ungated cells possessed the above amount of DNA. Cell populations with a DNA content of 4N through 32N were identified by the fluorescence intensity of the bound Hoechst dye. The cell populations of differing ploidy which exhibited β-galactosidase activity were sorted, and then stained for the lineage specific megakaryocyte marker, acetylcholinesterase, as previously described. Burstein, S. A. et al., J. Cell Physiol. 109:333-341 (1981).

The various organs, bone marrow cells, and platelets were quantitatively assayed for β- galactosidase activity. To this end, the cells and organs were solubilized by adding 0.1 to 0.4 gm of tissue to 2-3 ml of 1mM EDTA, 5 mM dithiothreitol, and 7% glycerol in 20 mM Tris buffer, pH 7.4 and then homogenizing the mixtures on ice with a Polytron homogenizer (PCU-2; Brinkmann Instruments). The homogenates were centrifuged at 4°C for 20 min at 110,000xg, supernatants were collected, and then

assayed for protein content by the method of Lowry et al. Lowry, O.H. et al., J. Biol. Chem. 193:265-275 (1951). Alternately, bone marrow cells or platelets were lysed with PM solution [60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCL; 1 mM $MgCl_2$; 50 mM B-mercaptoethanol] supplemented with 0.02% NP-40. The lysates were centrifuged at 4°C for 15 min in a microcentrifuge, the supernatants were collected, and then assayed for protein content as outlined above. The amounts of enzymatic activity present in the above samples were determined by adding 0.01-0.05 ml of the supernatant (30 ug of solubilized protein) to 0.8 ml of PM solution and 0.2 ml of 0-nitrophenyl galactopyranoside (gm/ml) (ONPG), and then monitoring the absorbance at 420 nm after varying lengths of time at 37°C. For each time point, the value obtained for a given nontransgenic tissue homogenate was subtracted from the value obtained for the same transgenic tissue extract. The concentrations of β-galactosidase in megakaryocytes were calculated by multiplying whole bone marrow values by 120 fold. This correction factor is derived from the relative frequency of megakaryocytes within the bone marow, and the relative size of this polyploid cell as compared to other cell types (see below). No attempt was made to directly quantitate the levels of β-galactosidase in purified megakaryocytes because of the small amounts of bone marrow available from each mouse, the low frequency of this cell type within the bone marrow, and the relative insensitivity of the assay for the prokaryotic enzyme with one unit of activity defined as an increase of 0.1 absorbance

units/hr at 420 nm. Prior experiments demonstrated that the homogenization procedure has minimal effect on β-galactosidase activity since the calculated recovery of purified enzyme added during the initial solubilization is greater than 90%.

Southern and Northern Blot Analysis

For Southern analysis, high molecular weight DNA from mouse tail segments was purified by phenol extraction and ethanol precipitation, digested with EcoRI, and quantitated by absorbance at 260 nm. Approximately 10 ug of each sample was fractionated by 1% agarose gel electrophoresis, transferred to a nitrocellulose filter, and hybridized with a ^{32}P labelled 3.34 kb prokaryotic β-Galactosidase DNA fragment. For Northern analysis, total RNA was isolated as previously described (Chomczynski, P. and Sacchi, M., Anal. Biochem. 162:156-159 (1987)), and then quantitated by absorbance at 260 nm. Approximately 10 to 30 ug of RNA per lane were then electrophoresed on 1% agarose/formaldehyde gels, blotted onto GENENTRAN nylon membranes (Plasco Inc., MA), and hybridized with ^{32}P labelled probe which is specific for rodent PF4 mRNA. After washing, the membranes were examined by Betascope (Betagen, Co., Waltham, MA) or exposed to x-ray film.

The offspring of the founder mice were investigated for expression of β-galactosidase in peripheral blood cells, bone marrow progenitor cells, splenic cells, and thymic cells. The results described below were obtained with line 10 transgenic mice but identical data were

generated with line 12 transgenic mice. Blood smears were prepared from transgenic mice as well as normal litter mates, and then examined by in situ staining for β-galactosidase utilizing X-Gall as substrate as outlined in Methods. Figure 2A shows that small, anucleated platelets of transgenic animals but not their normal litter mates stain for β-galactosidase whereas red cells and white cells from both types of mice do not contain the prokaryotic enzyme. To pinpoint transgene expression, blood from the two types of mice was processed to obtain fractions containing either platelets or red cells and nucleated white cells. Figures 2B and 2C reveal that platelets from transgenic mice stain intensely for β-galactosidase whereas those from normal itter mates do not possess the prokaryotic enzyme. Examination of the red cell/nucleated white cell fractions from both types of mice showed no staining for β-galactosidase. To ascertain whether transgene expression specifically takes place in megakaryocytes, bone marrow cells were prepared from both types of mice as described above and then examined by in situ staining for β-galactosidase. Figures 2D and 2E indicate that 0.05% to 0.10% of the nucleated bone marrow cells of transgenic mice but not their normal litter mates stain positive for β-galactosidase and exhibit diameters that range from about 20 microns to about 70 microns (normal hemopoietic cell diameters are 10 to 15 microns). Th frequency and size of these β-galactosidase positive cells are typical of bone marrow megakaryocytes.

The presence of PF4lacZ expression in bone marrow megakaryocytes and the absence of transgene function in

other hematopoietic cell lines was also confirmed. To this end, transgenic bone marrow cells were stained for β-galactosidase with the fluorescent substrate fluorescein-di-galactoside as well as for DNA content with Hoechst dye, and then subjected to flow cytometry as outlined in Methods. Based upon two separate experiments, we demonstrated that 0.1% of the transgenic bone marrow cells contained prokaryotic β-galactosidase with 26.9% of the total enzymatic activity in the 2N peak, 16.8% of the total enzymatic activity in the 4N peak, 10.1% of the total enzymatic acitivity in the 8N peak, 43.8% of the total enzymatic activity in the 16N peak and 2.4% of the total enzymatic activity in the 32N peak. The β-galactosidase positive cells were sorted and then stained for the rodent megakaryocyte specific enzyme, acetylcholinesterase. Burstein, A. S. et al., Blood 54:169-179 (1979). All cells including those obtained from the 2N peak possessed this lineage specific marker which confirms that bone marrown megakaryocytes selectively express PF4lacZ.

The presence or absence of transgene expression in various types of lymphocytes was also assessed. For this purpose, samples of spleen and thymus were obtained from transgenic mice and their normal litter mates. The tissues were fixed in paraformaldehyde; serial 6 micron thin sections of the various tissues were prepared, and then alternate sections were stained with hematoxylin and eosin or for β-galactosidase activity as outlined above. Figures 2F and 2G depict the results obtained with the spleen and thymus of transgenic mice. It is readily

apparent that large β-galactosidase positive cells are present within the spleen at an extremely low frequency. These cells were identified morphologically as megakaryocytes in sections stained with hematoxylin and eosin. The β-galactosidase positive cells were not observed in the spleen of nontransgenic mice. The thymus of transgenic mice exhibited no reaction for the prokaryotic enzyme, which was identical to that observed with their normal litter mates.

The possibility that the PF4lacZ transgene might be expressed in cells other than platelets and megakaryocytes was also assessed. The results described below were obtained with line 10 transgenic mice, but identical data were generated with line 12 transgenic mice. To this end, adrenal gland, brain, heart, intestine, kidney, liver, lung, and skeletal muscle were isolated from transgenic mice as well as from normal litter mates. The tissues were processed and examined by in situ staining for β-galactosidase as outlined above. The organs tested showed no staining for β-galactosidase, except for the consistent intense reaction of the transgenic adrenal cortex and the occasional weak reaction of the transgenic and nontransgenic endocardium. Figure 3 shows the β-galactosidase staining of the transgenic adrenal cortex; no such reaction was observed in the same organ from a normal litter mate. It is important to note a gradient of staining from the zona glomerulosa (most obvious) to the zona fasciculata (much less apparent) to the zona reticularis (minimally present), with a complete absence of reaction in the adrenal medulla. On rare

instances, weak scattered staining was observed in the transgenic endocardium, but was also occasionally seen in the endocardium of normal litter mates.

The relative levels of PF4lacZ transgene expression in the various organs were quantitated by kinetic (initial rate) assay of tissue homogenates for β-galactosidase activity per given amount of protein utilizing O-nitrophenyl galactopyranoside as substrate as outlined above. Based upon two separate experiments with line 10 mice, it was determined that megakaryocytes contain 6.00 units of β-galactosidase activity/30 ug of protein and that adrenal glands contain 0.11 units of β-galactosidase activity/30 ug of protein. The brain, heart, intestine, kidney, liver, lung, skeletal muscle, spleen, and thymus possess no detectible amounts of prokaryotic enzyme. Thus, transgene expression in megakaryocytes is about 50 fold greater than in adrenal gland, and at least 200 fold above other organs. The levels of β-galactosidase in the adrenal gland confirm the unexpected expression of the transgene in the endocrine organ. The quantitative results outlined above are in excellent agreement with the more qualitative observations made with in situ staining. For example, note the intense staining of megakaryocytes, the readily apparent but greatly reduced in situ staining of adrenal gland (Figure 3A), and the complete absence of in situ staining for other organs.

The above data suggest that the PF4 gene might be transcribed at low levels within the adrenal gland. To examine this issue, Northern analyses were carried out on

mouse or rat adrenal gland. They revealed a faint band of the same size as PF4 mRNA. Examination of other organs such as kidney also showed a barely detectible band in the same region. Northern analyses of varying concentraions of platelets suggested that the trapping of small numbers of these blood cells within the circulatory system of the various organs could explain the above observations. The use of other megakaryocyte specific DNA probes, such as GpIIb, to correct for potential platelet contamination proved fruitless because of the relatively low levels of expression of these genes vis a vis PF4. Thus, it was not possible to prove that the PF4 gene is actively transcribed within the adrenal gland. However, it is possible that the megakaryocyte message is produced at low levels similar to that contributed by platelets trapped within the circulatory system of this endocrine organ. Alternately, the adrenal gland may express transacting factors similar to those which control the megakaryocyte promoter.

CLAIMS

1. A method of selective targeting of a gene product in an animal to platelets and bone marrow megakaryocytes, comprising introducing into the animal a megakaryocyte promoter-nucleic acid sequence construct comprising a nucleic acid sequence encoding the gene product under the control of a megakaryocyte promoter, under conditions appropriate for expression of the nucleic acid sequence encoding the gene product in platelets and bone marrow megakaryocytes.

2. The method of Claim 1 wherein the megakaryocyte promoter-nucleic acid sequence construct is a platelet factor 4 promoter-nucleic acid sequence construct and is introduced into the pronucleus of an embryo.

3. The method of Claim 2 wherein the platelet factor 4 promoter is a rat platelet factor 4 promoter 1.1 kb in size and the nucleic acid sequence encodes β-galactosidase.

4. The method of Claim 1 wherein the megakaryocyte promoter-nucleic acid sequence construct is a platelet factor 4 promoter-nucleic acid sequence construct and is introduced into bone marrow.

5. A megakaryocyte promoter-nucleic acid sequence construct wherein expression of the nucleic acid sequence is under the control of the megakaryocyte promoter.

6. The construct of Claim 5 wherein the megakaryocyte promoter is the platelet factor 4 promoter.

7. The construct of Claim 6 wherein the nucleic acid sequence is the β-galactosidase gene.

8. The plasmid designated PF4lacZ, deposited at the American Type Culture Collection under Accession Number 40903.

9. A method of producing a selected product in platelets and bone marrow megakaryocytes comprising introducing into the platelets or the bone marrow megakaryocytes a megakaryocyte-promoter-nucleic acid sequence construct in which expression of the nucleic acid sequence is under the control of the megakaryocyte promoter, under conditions appropriate for expression of the nucleic acid sequence.

10. A method of producing an immortalized megakaryocyte cell line, comprising the steps of:

a. introducing into a fertilized egg of an animal a megakaryocyte promoter-oncogene construct, in which the oncogene is a temperature sensitive mutant, under conditions appropriate for expression of the oncogene in megakaryocytes;

b. obtaining bone marrow from the animal; and

c. culturing the bone marrow at a temperature permissive for the oncogene, thereby producing an immortalized megakaryocyte cell line.

11. A transgenic non-human mammal in which platelets, bone marrow megakaryocytes or both contain a product encoded by a nucleic acid sequence intoduced, as a component of a megakaryocyte promoter-nucleic acid sequence construct, into the pronucleus of an embryo from which the transgenic mammal, or an ancester of the transgenic mammal, developed.

12. The transgenic non-human mammal of Claim 11 in which the product is introduced into the promucleus of an embryo as a component of a platelet factor 4-nucleic acid sequence construct.

13. The transgenic non-human mammal of Claim 12 wherein the transgenic non-human mammal is a mouse.

14. A transgenic non-human mammal in which platelets, bone marrow or both contain a product encoded by a nucleic acid sequence introduced into the non-human mammal, or an ancestor of the non-human mammal, at an embryonic stage.

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-181 AAGCTTAAGA AATCGTAAGA CTTGTCTTGG GTTAGAAGTG TAGCTCAGTG
     GTAGAATGCT TGCCCAGCAC GTGAGAGGCC CTAGGTTCAA TTCCCAGCAG
-171 AGTCCAAAGA AACCTGCCTT AGAAAGTAAA TGACCTGAGT GCATATTGGA
      AACCATGCTC GTAAAGCACT AGAGCTTGAC CCTTTTGCTG ATCCTTCCTT
-161 CCCTAAGAGG CCACTTCACA TCACATCAGT TATAGGTACA TGGGATACTG
      CTTGATTCAA ACTCCCTACT CTATCCTAGT CATGGAAGAA GAGATAATTT
-151 CACCCCCTCC AACCTGTACA TGGGCTTTGG GGCACTCGTA TACGCTAAAC
      ACGTACTTCA CTCAAGACTC AGCATGATGG AGGGTTTCCT TTTGTCTCTT
-141 CACCACCAGA GGCTCAGGTC TGCACACATC TTGTCTGTAA TTCAGTTGTA
      ACTACTCAAA TGTCCACAAC CATGTGAGAA AGCAGTAAAC GGTTTTATTA
-131 GCTTCGCTTC AGTTACTATG ACAGCTGTGC TCTCTGAGGC GGGTCTCTCT
      ACCGAAAGCT ATCAAGGCTG GTTCTTACCA CAACACTGGT TGCCCTGTGC
-121 NTTTCTTGAG GTGAACGTAT CTCTCACTGC TGGGCACCCA AACTGGATTA
      TCTTGATTAT CTTCTTTAAA GCTAGAGAAA GGCTGCCTTG CCTGACATCA
-111 TTGCTTGAAT TCCTTTACTC TGCGAATGCT GAAATCTTTG GTGAAGGTGG
      CACAGAAGAG TTTTCTTGCT GTCCAGATTA AAATCCTCTT ATCATATATA
-101 TATGTATATA TATATATATA TATATATATA TATTTTTTTT TTTTTTTTTT
      TTTTTTTTGC TGACCCAGCC GAGGCCTTGA GTTTCAGTTC CCTAAAGGCA
-91 TAGGGATTCT GACATGTTTT GCAGTAGCCG TTGTTGTCCA TGCTGAGTGT
      CCATAAATGT ATGCCCCGGG GAGTTATGCT TGACTATATC ACTATGCATG
-81 GGGGGCAGAT AAAGGGGCAC ATAATTATAA CCATATATGG CATGAATTAA
     TATAGAGATC TCCAACTGTC CCAGAAAGCT TTCTTCTCTA CTCTTCTCCA
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Figure 1A

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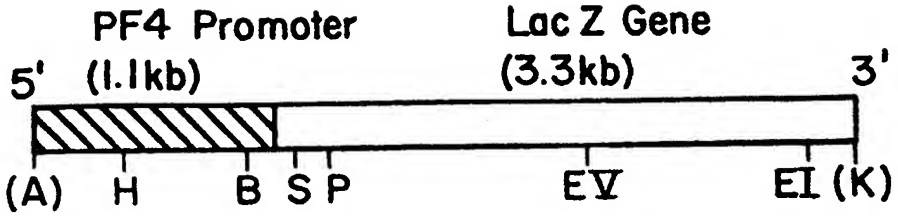
-711 GGGTAGAGCT GAGCAGACTA AAAGATTTTA TCAAAGAAAA GCCTTCAAAT
     ACTACCTCAG GGATGTTTCT AAAGAGTCCT GAGAAGAGCA GACCCTGCCG
-611 CCTCCAGTAG ATTGGACAGC CGTAGCTCAG AAGAGCCTCC AGAATTTCCT
     GCAGGAGGCT TCAGAAGGTT TCCATCGTGA GGATGAAAGT CAGAAGCCAT
-511 TGCCTAGCCA TTCACTTCAA TGTTTTAATG GCAGAAAATA AATTTCCAGT
     CACGCATCTC TAACCACATG GCAGTCAAAC TCACAGCAAA TCAACAGGAA
-411 AGCACGGCAG GGTGTTTGGG GTCGGGCAAC CGGAAGTCGG GAAGGCAACA
     AATTGGTACT GAAGGTGCAT GTTCTGTAAA CCGCATGGGG ATAGCAGAAA
-311 ATTCTCTGCC ACATACAGCA TACCTTCTGC GAAAATTCCA ACTGTTTCTA
     CCTCTGTAGA CTGTTCACAT AAATTCACAT TGGGGACGTG GATCCTGCTG
-211 ACAGCTGCTG ACAGCTGGCC TCAGCTGCTT CTTTTTTTTT TTTTTTTTT
     TTTTTTTCCT GTTCTATGTT GCTTTAATCT TGGCTGGCCA GATCTCAAGT
-111 ACTGTTCCAC AAGTGTCATT GCTTCTGTGG ATCACTTCCT CATCCCCTAT
     CCCGGGTTTC CGGACTGGGC TGGCAGTGAA GATAAAACGT GTCTAGAAAG
  - 11 TCACAGGAGC CACTGTCTGG CACTTAGAGC CCCAGACCCC AGTTTCCCCG
```

Figure 1B

FIG. 2A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Lac Z Gene
(3.3kb)
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3'
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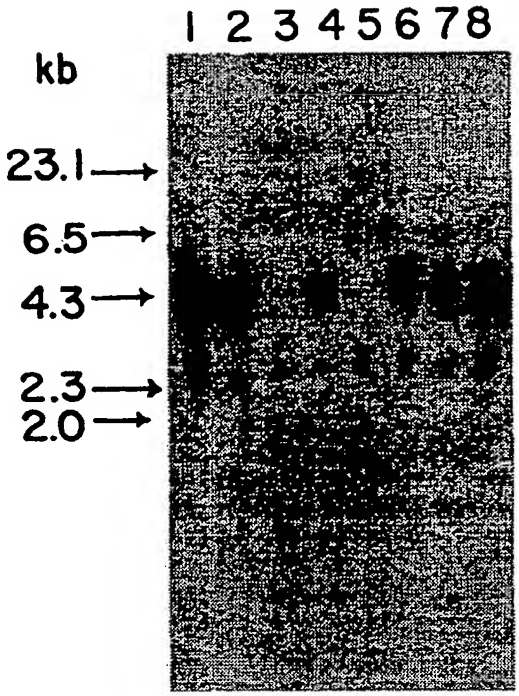
B
S
P
EV
EI
(K)

FIG. 2B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kb
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6.5
4.3
2.3
2.0

FIG. 3A

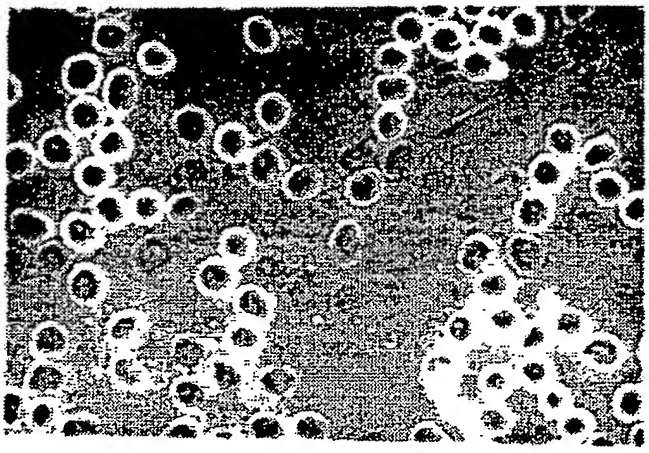


FIG. 3B

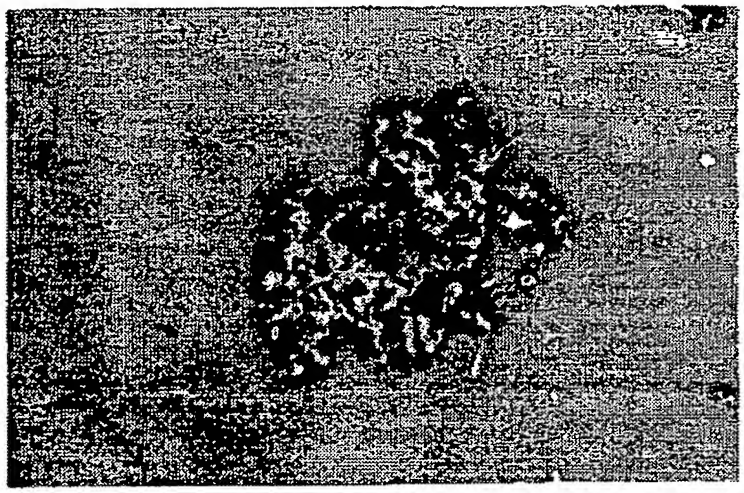


FIG. 3C

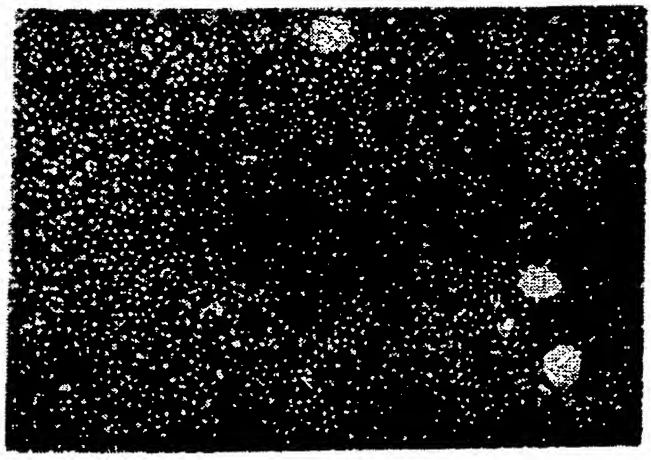


FIG. 3D

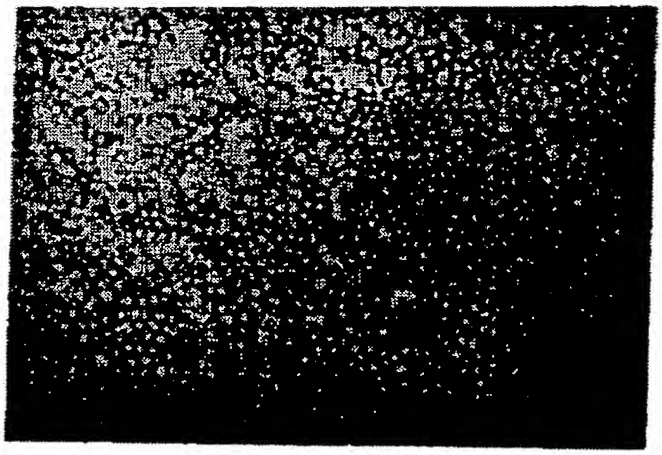


FIG. 3E

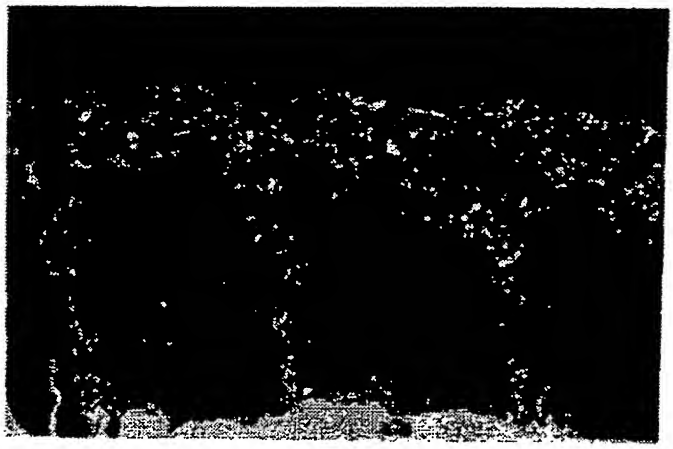
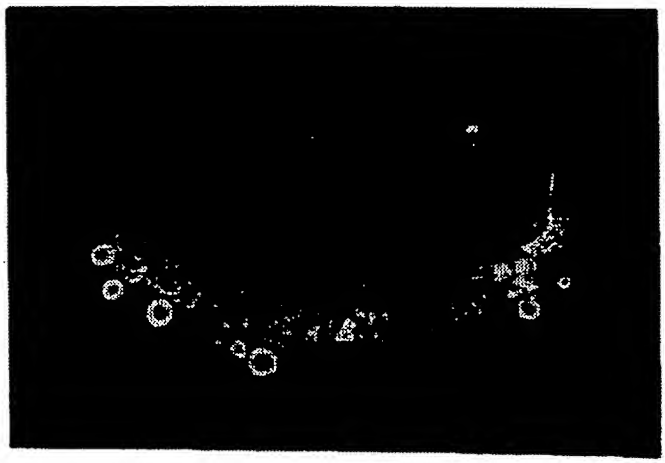


FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/07233

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; C12N15/11; A61K48/00; C12N5/10
-- C12N15/85; A01K67/027

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	FASEB JOURNAL. vol. 4, no. 7, 26 July 1990, BETHESDA, MD US RAVID, K. ET AL.: 'tRANSCRIPTIONAL REGULATION OF THE RAT PLATELET FACTOR 4 GENE' cited in the application	1,5-9
Y	see abstract 1392	4,9, 10-14
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 4, February 1991, WASHINGTON US pages 1521 - 1525; RAVID, K. ET AL.: 'Selective targetting of gene products with the megacaryocyte platelet factor 4 promoter'	1-3,5-8, 11-14
P,Y	see the whole document	4,9, 10-14

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 FEBRUARY 1992	27.02.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

2

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	MOLECULAR AND CELLULAR BIOLOGY vol. 11, no. 12, December 1991, pages 6116 - 6127; RAVID, K. ET AL.: 'Transcriptional regulation of the rat Platelet Factor 4 gene: interaction between an enhancer/ silencer domain and the GATA site' see the whole document	1,5-6,9 4,10
X	NATURE. vol. 318, 12 December 1985, LONDON GB pages 533 - 538; ADAMS, J.M. ET AL.: 'The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice' see table 1	14
Y	see the whole document	10
X	TRENDS IN GENETICS vol. 9, 1985, AMSTERDAM, NL pages 231 - 236; ALT, F.W. ET AL.: '<immunoglobulin genes in transgenic mice' see specially figure 2 see the whole document	14
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, December 1988, WASHINGTON US pages 9037 - 9041; EFRAT, S. ET AL.: 'beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene' see the whole document	10
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, no. 1, January 1990, WASHINGTON US pages 439 - 443; WILSON, J.M. ET AL.: 'Expression of human adenosine deaminase in mice reconstituted with retrovirus-transduced hematopoietic stem cells' see the whole document	4,9